Biochimica et Biophysica Acta 1833 (2013) 3093–3103

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

DNA damage response (DDR) induced by topoisomerase II poisons requires nuclear function of the small GTPase Rac

Friedrich Wartlick, Anita Bopp, Christian Henninger, Gerhard Fritz *

Institute of Toxicology, Heinrich-Heine-University Düsseldorf, Moorenstrasse 5, D-40225 Düsseldorf, Germany

ARTICLE INFO

Article history:
Received 31 May 2013
Received in revised form 14 August 2013
Accepted 23 August 2013
Available online 30 August 2013

Keywords:
Rac family small GTPase
Topoisomerase II poisons
DNA damage response

ABSTRACT

Here, we investigated the influence of Rac family small GTPases on mechanisms of the DNA damage response (DDR) stimulated by topoisomerase II poisons. To this end, we examined the influence of the Rac-specific small molecule inhibitor EHT1864 on Ser139 phosphorylation of histone H2AX, a widely used marker of the DDR triggered by DNA double-strand breaks. EHT1864 attenuated the doxorubicin-stimulated DDR in a subset of cell lines tested, including HepG2 hepatoma cells. EHT1864 reduced the level of DNA strand breaks and increased viability following treatment of HepG2 cells with topo II poisons. Protection by EHT1864 was observed in both p53 wildtype (HepG2) and p53 deficient (Hep3B) human hepatoma cells and, furthermore, remained unaffected upon pharmacological inhibition of p53 in HepG2. Apparently, the impact of Rac on the DDR is independent of p53. Protection from doxorubicin-induced DNA damage by EHT1864 comprises both S and G2 phase cells. The inhibitory effect of EHT1864 on doxorubicin-stimulated DDR was mimicked by pharmacological inhibition of various protein kinases, including JNK, ERK, PI3K, PAK and CK1. EHT1864 and protein kinase inhibitors also attenuated the formation of the topo II-DNA cleavable complex. Moreover, EHT1864 mitigated the constitutive phosphorylation of topoisomerase IIα at positions S1106, S1213 and S1247. Doxorubicin transport, nuclear import/export of topoisomerase II and Hsp90-related mechanisms are likely not of relevance for doxorubicin-stimulated DDR impaired by EHT1864. We suggest that multiple kinase-dependent but p53- and heat shock protein-independent Rac-regulated nuclear mechanisms are required for activation of the DDR following treatment with topo II poisons.

1. Introduction

Small GTPases of the Rho (Rho = Ras homologous) family play pivotal roles in the regulation of a variety of cellular functions, including proliferation and gene expression as well as regulation of the actin cytoskeleton and apoptosis [1–3]. Among this family, Rac1 is a key regulator of genotoxic stress-induced activation of stress-activated protein kinases/c-Jun-N-terminal kinases and p38 kinases [4,5] and related transcription factors including NF-kB and AP1 [6,7]. Due to the pleiotropic functions of Rho GTPases in tumor development and progression, targeting of Rho functions is suggested as therapeutic strategy to improve anticancer therapy [8,9]. Rho-regulated mechanisms do not only promote the anticancer efficacy of conventional anticancer drugs [10] but also attenuate adverse effects of ionizing radiation and topoisomerase II inhibitors on normal tissue [11,12]. Therefore, targeting of Rho signaling might be a promising strategy to widen the therapeutic window of anticancer therapeutics. One therapeutic option to target Rho signaling are HMG-CoA reductase inhibitors (statins), which deplete the cellular pool of isoprene precursor molecules, which are essentially required for Rho function [13]. Most of the pleiotropic functions of statins, including inhibition of cell proliferation [14], induction of cell death [15], antimetastatic effects [16], increase in the therapeutic efficacy of a variety of conventional anticancer drugs [17,18] and protection from anticancer therapy-induced normal tissue damage [11,12] have been attributed to interference with RhoA and Rac1 [19,20].

Inhibition of topoisomerase II (topo II) by anticancer drugs such as the anthracycline derivative doxorubicin and the podophyllotoxin etoposide results in the formation of DNA double-strand breaks (DSBs) [21,22]. DSBs are potent activators of the DNA damage response (DDR) and trigger cell death [23–25]. Phosphorylation of histone H2AX on S139 (γH2AX) is a well accepted surrogate marker of the DDR induced by DSBs [22,24,26]. The clinical use of topo II poisons is limited by adverse effects, in particular cardiotoxicity in case of doxorubicin [27,28], hematologic toxicity in case of etoposide [29] and, for both of them, secondary leukemia [29,30]. Regarding anthracyclines, redox cycling leading to oxidative stress and the inhibition of type II topoisomerases (i.e. topo IIα and topo IIβ) are controversially discussed as critical events responsible for their cardiotoxicity [28,31]. Recently it was shown that mice lacking cardiac expression of topo II beta are protected from doxorubicin-induced cardiac injury [32], indicating that inhibition of this topo II isoform by doxorubicin is most relevant for cardiotoxicity. Regarding Rac1, it was suggested that this GTPase mediates doxorubicin-induced cardiotoxicity through both ROS dependent and independent pathways [33]. Targeting

* Corresponding author. Tel.: +49 211 8113022; fax: +49 211 8113013.
E-mail address: fritz@uni-duesseldorf.de (G. Fritz).

0167-4889/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamcr.2013.08.016

of Rho signaling, for example by HMG-CoA reductase inhibitors (statins) [10,13,20], has multiple inhibitory effects on stress responses following exposure of normal cells to anticancer therapeutics. For instance, statins inhibit the activation of the DDR of human endothelial cells (HUVEC) and smooth muscle cells stimulated by ionizing radiation [34,35] and, moreover, attenuate doxorubicin- and etoposide-induced stimulation of the DDR in HUVEC and rat cardiomyoblasts (H9c2) *in vitro* [34,36,37]. Besides, beneficial effects of statin-mediated inhibition of Rho-signaling on normal tissue damage provoked by anthracyclines and ionizing radiation have been reported [38,39]. Recently, binding of Rac1 to topoisomerases has been demonstrated [40], suggesting that Rac1 might have an additional nuclear function, which is related to topoisomerases. In the present study we investigated the influence of Rac-regulated mechanisms on the complex network of DNA damage-triggered stress responses evoked by the topoisomerase II poisons doxorubicin and etoposide. To this end, the Rac-specific small-molecule inhibitor EHT1864 was employed [41,42].

2. Materials and methods

2.1. Materials

The following antibodies were used in our study: γH2AX (pS139), H2AX and Rac1 were from Millipore (Billerica, MA, USA), γH2AX (pS139), topo IIα (pS1106), GAPDH, lamin B2, Chk2 (pThr68) and Chk2 were obtained from Epitomics (Burlingame, CA, USA). ATM (pS1981), Histone H3 (pS10), Akt (pS473), Akt, p38 (pThr180/pTyr182), p53 (pS15), NF-κB subunit p65, cleaved caspase-7 and BRCA1 originate from New England Biolabs (Frankfurt, Germany), survivin, cyclin B1, ERK2, p38 and JNK (pThr183/pTyr185) were from Santa Cruz (CA, USA). Topo IIα was provided by Enzo Life Sciences (Lörrach, Germany), topo IIβ by Abcam (Cambridge, MA, USA), p53 by Exbio Praha (Prague, Czech Republic), HSP90 by Cayman Chemical Company (MI, USA), HSP70 by Biosciences Inc. (Allentown, PA, USA), β-Actin and JNK by Sigma Aldrich (Hamburg, Germany), and Alexa Fluor 488 by Invitrogen (Paisley, UK). HRP conjugated secondary anti-mouse antibody and HRP conjugated secondary anti-rabbit antibody were from Rockland Immunochemicals (Gilbertsville, PA, USA).

The inhibitors used were purchased from the following providers and used at the concentrations indicated: Rac1 small molecule inhibitors EHT1864 (10 μM) and NSC23766 (100 μM), JNK inhibitor SP60025 (10 μM), PAK inhibitor IPA-3 (10 μM) (Tocris, Bristol, UK), p38 inhibitor SB203580 (10 μM), ERK inhibitor PD98059 (10 μM) (Merck Millipore, Darmstadt, Germany), Akt inhibitor 1L6-Hydroxymethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecyl-*sn*-glycerocarbonate (5 μM), BCR-Abl inhibitor II (10 μM), CDK1 inhibitor 3-(2-Chloro-3-indolylmethylene)-1,3-dihydroindol-2-one (10 μM) (Merck Calbiochem, Darmstadt, Germany), Hsp90 inhibitor geldanamycin (10 μM), PI3K inhibitor 3MA (10 μM) (InvivoGen, Toulouse, France), p53 inhibitor pifithrin-α (30 μM), protein kinase CK1 inhibitor IC261 (10 μM and 30 μM), CK2 inhibitor resorufin (30 μM), Scr-family tyrosine kinase inhibitor PP2 (10 μM), nuclear import inhibitor Ivermectin (25 μM) (Sigma-Aldrich, Hamburg, Germany), NF-kB inhibitor SC-3060 (10 μM), nuclear export inhibitor ratjadone C (10 μM) (Santa Cruz, CA, USA), EGF receptor inhibitor Iressa (10 μM) (AstraZeneca, London, UK). Cell permeable Rac1 inhibitory peptides comprising the amino acid sequence of the Rac1 effector binding region (Rac1-Switch I domain (AA 24–45)) (10 μM), the sequence of Rac1 to which GTPase activating protein (GAPs) binds (Rac-GAP domain (AA 74–90)) (10 μM) or of the CRIB domain of the Rac1 effector p21-associated protein kinase (PAK1) (PAK1-CRIB domain (AA 70–94)) (10 μM) were synthesized by JPT Peptide technology GmbH (Berlin, Germany). The peptides contain a C-terminal HIV-TAT in order to enable uptake (HIV-TAT/Rac-SwitchI (24–45): YGRKKRRQRRR-PP-NAFPGEYIPTVFDNYSANVM; HIV-TAT/Rac-GAP (74–90): YGRKKRRQRRR-PP-QTDVFLICFSLVSPASF HIV-TAT/PAK1-CRIP (70–94): YGRKKRRQRRR-PP-KERPEISLPSDFEHTIHVGFDAVTG). The topo II poisons doxorubicin and etoposide were provided by the pharmaceutical department of the University Medical Center of the Heinrich Heine University Düsseldorf.

2.2. Cell culture and drug treatment

Human hepatoma cells (HepG2 and Hep3B), rat hepatoma cells (H4IIE), human colon carcinoma cells (HCT116 and HT29), human embryonic kidney cells (HEK293) and rat kidney epithelial cells (NRK-52E) were grown at 37 °C in DMEM + 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Immortalized human endothelial cells (EA.hy926) were grown at 37 °C in DMEM + 10% fetal calf serum + HAT supplement (Sigma-Aldrich, Hamburg, Germany) and the aforementioned antibiotics. Primary human umbilical vein endothelial cells (HUVEC) were grown at 37 °C in ECGM2 (PromoCell, Heidelberg, Germany) plus antibiotics. Human DLD1 carcinoma cells and rat kidney endothelial RGE cells were grown at 37 °C in RPMI supplemented with 10% fetal calf serum plus antibiotics. Human ovarian cancer cells (A2780) were cultured at 37 °C in RPMI supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics. If not stated otherwise, treatment of logarithmically growing cells with topoisomerase inhibitors was performed for 4 h. Pre-treatment with Rac-specific small molecule inhibitor EHT1864, which causes guanine nucleotide displacement, prevents nucleotide exchange by guanine exchange factors (GEFs) and inhibits downstream signaling [41,42], was performed for 1 h.

2.3. Preparation of cell extracts

For preparation of total cell extracts identical number of cells were lysed in 300 μl Roti®-Load 1 sample buffer (Roth, Karlsruhe, Germany). After sonication (EpiShear™ (2 mm), Active Motif, Carlsbad, CA, USA), extract was incubated for 5 min at 95 °C. After centrifugation (5 min, 14,000 ×*g*, RT) the supernatant was used for western blot analysis. For preparation of nuclear and cytosolic extracts, about 2×10^6 cells were lysed in 300 μl buffer containing 10 mM Tris/HCl (pH 7.5), 50 mM NaCl, 0.5 M Sucrose, 0.1 mM EDTA, 0.5% Triton-X 100, 1 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4 and a protease inhibitor mix (Roche, Mannheim, Germany). Extracts were incubated for 5 min on ice before centrifugation (10 min, 1000 ×*g*, 4 °C). The supernatant, which contains cytosolic proteins, was centrifugated again (15 min, 14,000 ×*g*, 4 °C) to remove floating particles before Roti®-Load1 sample buffer was added. The pellet was used for isolation of nuclear proteins. To this end, it was resuspended in buffer (about 4× the volume of the pellet) containing 10 mM Tris/HCl (pH 7.5), 500 mM NaCl, 0.2 mM EDTA, 0.1% NP-40, 0.1% tergitol, 1 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4 and a protease inhibitor mix. After sonication (5 times for 1 s, ampl. 25%), samples were incubated for 5 min on ice before Roti-load sample buffer was added.

2.4. Western blot analysis

Proteins were separated by SDS-PAGE (7.5–15%), transferred onto a nitrocellulose membrane. After blocking in 5% non-fat milk in TBS/0.1% Tween for 1 h at RT, incubation with the primary antibody was performed over night at 4 °C. After washing, incubation with peroxidase conjugated secondary antibody (1:2000) was performed for 2 h at RT. For visualization of the bound antibodies Fusion FX7 imaging system (Peqlab, Erlangen, Germany) was used.

2.5. Immunoprecipitation experiments

Cells were lysed in 300 μl lysis buffer (25 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol, 1 mM PMSF, 1 mM Na_3VO_4, protease inhibitor mix). After sonication and centrifugation (2 min, 14,000 ×*g*), 50 μl of the supernatant was taken for input control (done

by western blot). For immunoprecipitation, 1 μg antibody was added to the supernatant, followed by incubation for 1–2 h at 4 °C on a rotating shaker. 100 μl of A/G-Beads (Santa Cruz, CA, USA), which were calibrated in lyses buffer, was added to the cell lysate. After incubation over night on a rotating shaker at 4 °C, beads were pelleted (1 min, 1000 ×*g*) and washed 3 times with lysis buffer. Afterwards 1× Roti-load sample buffer (Roth, Karlsruhe, Germany) was added, followed by incubation for 15 min at 37 °C (gently shaking) and heating for 5 min 95 °C. After centrifugation, the supernatant was subjected to western blot analysis.

2.6. Analysis of H2AX phosphorylation by FACS

Quantitative evaluation of the level of Ser139-phosphorylated H2AX, which is indicative of DNA damage [24], was done either by western blot analysis or by FACS-based analysis. For FACS-based quantitation of γH2AX levels, cells (about 2×10^6) were trypsinated, washed with PBS (300 ×*g*, 4 min) and resuspended in 0.5 ml PBS. 4.5 ml ice cold 1% methanol free formaldehyde solution was added and the suspension was incubated on ice for 15 min. After washing with PBS (300 ×*g*, 4 min) and resuspending in 0.5 ml PBS, the suspension was added to 4.5 ml −20 °C cold 70% ethanol and incubated at −20 °C for at least 2 h. Afterwards, cells were pelleted (200 ×*g* for 4 min), ethanol was removed, the cell pellet was washed with BSA-TPBS solution (1% BSA in PBS Triton X-100 0.2% (v/v)), followed by incubation 2 ml BSA-TPBS for 5 min at RT. After centrifugation (300 ×*g* for 4 min) cells were resuspended in 100 μl BSA-TPBS containing 1 μg γH2AX antibody and incubated over night at 4 °C. After washing, 100 μl BSA-TPBS containing 1 μg FITC-tagged secondary antibody (Alexa Fluor 488) was added and incubated for 1 h at RT with occasionally shaking. After adding 5 ml of BSA-TPBS, incubation for 2 min at RT and centrifugation (300 ×*g* for 4 min), cells were resuspended in 1 ml of PI staining solution (PBS containing 100 mg/ml RNase A (Sigma-Aldrich, Hamburg, Germany) and 5 μg/ml propidium iodide). After incubation for 1 h at RT in the dark, the cells were analyzed via FACS.

2.7. Analysis of cell viability

Cell viability was analyzed using the water soluble tetrazolium salt (WST-1) assay according to the manufacturers' protocol (Roche Diagnostics, Mannheim, Germany). Briefly, the cell permeable tetrazolium salt is cleaved by the mitochondrial reductase system that is present in metabolically active (viable) cells, resulting in the formation of a formazan dye. Absorbance of this dye can be measured photometrically (max. absorption at 440 nm; reference wavelength 600 nm).

2.8. Analysis of DNA strand break induction (Comet assay)

Formation of DNA strand breaks was assayed by the alkaline comet assay [43]. Comets were visualized by microscopy and quantified by determination of the percentage of DNA in the tail (Komet 4.02, Kinetics Imaging, UK). 50 nuclei were evaluated per treatment.

2.9. Doxorubicin import/export analysis

To analyze doxorubicin transport, cells were treated with 1 μM of the anthracycline for 2 h. Afterwards, cells were trypsinized, washed with PBS and fixed with ice cold 70% ethanol for at least 1 h at −20 °C. The inherent doxorubicin autofluorescence was taken as indication for the amount of doxorubicin that has been taken up into the cell and was measured via FACS (excitation: 470 nm; emission: 575 nm). To calculate doxorubicin export, doxorubicin treated cells were post-incubated in doxorubicin free medium for 6 h before the residual intracellular amount of doxorubicin was determined by FACS.

2.10. Mass spectrometry analysis

After drug treatment, topo IIa protein was immunoprecipitated with topo IIα specific antibody as described above. Immunoprecipitated protein was separated by SDS-PAGE (7.5%) and the gel was stained with Coomassie Crystal Blue solution. The topo IIα protein band was cut out of the gel. Identification of phosphorylation sites was done by mass spectrometry-based analysis performed as service by the Molecular Proteomics Laboratory (Heinrich Heine University Düsseldorf).

2.10.1. Analysis of topo II-DNA complex formation by the band-depletion assay

The formation of the topo II-DNA cleavable complex was analyzed by the band-depletion assay [44]. High dose of etoposide (i.e. 100 μM) is used to trap active topo II protein to the DNA. Only non-trapped topo II can migrate into the polyacrylamide gel and is detectable by western blot analysis. Correspondingly, a decrease in topo II protein level as detected by western blot analysis is indicative of trapped (i.e. DNA bound) topo II enzyme. HepG2 cells were preincubated with EHT1864 or various protein kinase inhibitors for 2 h followed by treatment with 100 μM etoposide for 60 min. Cells were lysed with Roti-load sample buffer (Roth, Karlsruhe, Germany), sonicated and incubated for 5 min at 95 °C. After centrifugation (10 min, 14,000 ×*g*, RT) supernatants were subjected to western blot analysis (7.5% SDS polyacrylamide gel) using topo IIα and topo IIβ specific antibodies as well as α-tubulin and lamin B2 antibodies for protein loading control.

2.11. Statistical analysis

For statistical analysis the Student's T-test or the Mann–Whitney U-test was applied. p-Values ≤ 0.05 were considered significant and marked with an asterisk.

3. Results and discussion

3.1. The influence of Rac-regulated signaling mechanisms on doxorubicin-induced DDR is cell-type specific

In previous study we observed that inhibition of Rac signaling by statins results in an attenuated DDR of non-malignant endothelial cells (HUVEC) and rat cardiomyoblasts (H9c2) following treatment with the anthracycline derivative doxorubicin [12,34,37]. Here we addressed the question of whether the interference of Rac with mechanisms of the DDR simulated by topoisomerase II poisons is specific for non-malignant cells. To this end, we extended our previous studies by investigating the influence of the Rac specific small-molecule inhibitor EHT1864 [41,42] on doxorubicin-induced DDR in various hepatoma and colon carcinoma cell lines. EHT1864 was employed because in preliminary tests it was found to inhibit mitogen-stimulated activation of Rac1, as measured by GST-PAK-mediated pull-down of active (i.e. GTP-bound) Rac1 in HepG2 cells, more efficiently than NSC23766 (data not shown), which is another type of Rac inhibitor [45]. Liver and colon cells were chosen because these cell types are exposed to the highest concentration of statins and other Rac-inhibitory drugs after their oral intake. For additional control, a number of endothelial cell lines and cell lines from different origin were included as well. A total of 13 different cell lines of human and rodent origin were examined. To monitor the effect of EHT1864 on doxorubicin-induced DNA damage induction and mechanisms of the DDR, Ser139 phosphorylation of histone H2AX (γH2AX), which is a generally accepted surrogate marker of the DDR triggered by DNA double-strand breaks (DSBs) [22,24,26], was analyzed by western blot analysis. In addition, activation of p53, which is another hallmark of the DDR important for the regulation

of cell cycle progression, DNA repair and cell death [23], was analyzed by use of an antibody specifically detecting Ser15-phosphorylated p53 protein (pp53).

Out of three hepatoma cell lines analyzed, EHT1864 reduced the doxorubicin-stimulated increase in γH2AX levels only in the two human cell lines HepG2 and Hep3B, but not in rat H4IIE cells (Fig. 1A). Regarding activation of p53, EHT1864 decreased both the level of Ser15 phosphorylated p53 and the overall protein amount of p53 in HepG2 cells (Fig. 1A). Both Hep3B and H4IIE harbor mutated p53 and do not show p53 protein stabilization following doxorubicin exposure (Fig. 1A). Neither doxorubicin nor EHT1864 altered the expression levels of topo II isoforms (Fig. 1A). Next we investigated the expression of heat shock protein Hsp90 because it interacts with topoisomerase IIα [46], influences doxorubicin resistance [47] and, moreover, is also reported to interact with Rac1 [48]. Yet, as shown in Fig. 1A, EHT1864 did not change the level of Hsp90 protein and also not of Hsp70, which was included as control. The amount of Ser10 phosphorylated histone H3, which is a mitotic marker, decreased after doxorubicin exposure in the human hepatoma cell lines. This decrease was not rescued by EHT1864 (Fig. 1A). Regarding colon carcinoma cells, phosphorylation of H2AX stimulated by doxorubicin treatment was blocked by EHT1864 in DLD1 cells and, to a weaker extent, in HT29 cells (Fig. 1B). However, the Rac inhibitor did not mitigate the doxorubicin-stimulated increase of pp53 in colon cells (Fig. 1B). Regarding endothelial cells, attenuation of doxorubicin-stimulated increase in γH2AX and p53 by ETH1864 was detectable in primary human endothelial cells (HUVEC) only, but not in immortalized EA.hy926 cells or rat glomerular endothelial cells (RGE) (Fig. 1C). Other subset of human tumor cell lines also failed to respond to EHT1864, except MCF-7 mammary carcinoma cells (Fig. 1D). Taken together, the data show that the requirement of Rac signaling for the DDR, as reflected on the level of γH2AX and pp53, is cell-type specific and, apart from primary human endothelial cells [34] and rat cardiomyoblasts [12,37], also pertains to a subset of tumor cell lines, including human hepatoma cells. The data obtained with the various cell lines are summarized in Supplementary Table 1. The inhibitory effect of EHT1864 on doxorubicin-induced DDR of HepG2 hepatoma cells is not due to altered doxorubicin transport. FACS-based analysis of the intracellular doxorubicin concentration showed that neither influx nor efflux of doxorubicin is affected by EHT1864 (Supplementary Fig. S1). The molecular mechanism underlying the cell-type specificity of EHT1864 is unclear. It is conceivable that it is related to the Rac1 protein level because, with the exception of A2780 cells, cell lines harboring relatively high amount of Rac1 protein did not respond to EHT1864 co-treatment with an attenuated DDR stimulated by doxorubicin. An association between EHT1864 responsiveness and the constitutive level of S1106-phosphorylated topo IIα was not observed.

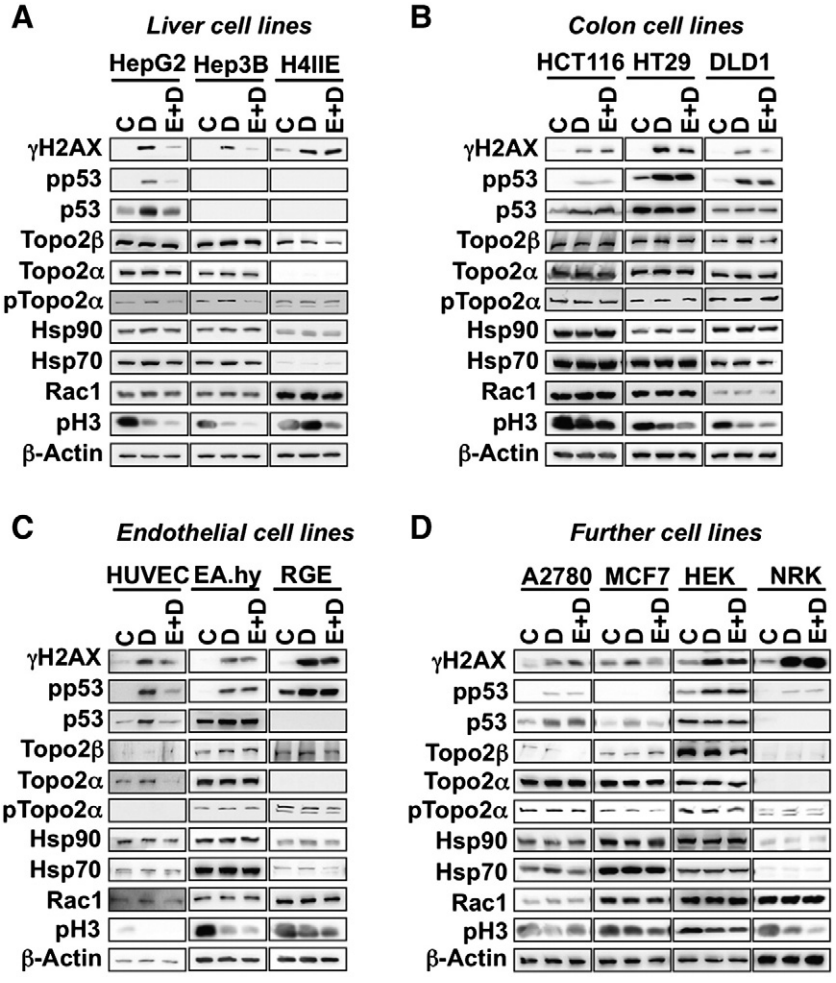

Fig. 1. Cell type-specific inhibition of doxorubicin-induced DDR by EHT1864, a small molecule inhibitor of Rac family GTPases. Logarithmically growing human and murine cell lines of different origin were pretreated or not (C = untreated control) with EHT1864 (10 μM) (E) for 1 h. Afterwards, the anthracycline derivative doxorubicin (D) was added (1 μM) and protein expression of the indicated proteins was analyzed after further incubation period of 4 h by western blot analysis. A, human (HepG2, Hep3B) and rat (H4IIE) hepatoma cell lines; B, human colon carcinoma cell lines; C, human (HUVEC, EA.hy926) and rat (RGE) endothelial cell lines; D, human ovarian (A2780) and breast carcinoma cells (MCF7), human embryonic kidney (HEK293) and rat tubular kidney (NRK-52E) cells.

3.2. EHT1864-mediated protection of human hepatoma cells from DNA damage induction and cytotoxicity provoked by topoisomerase II poisons is independent of p53

EHT1864-mediated inhibition of doxorubicin-induced DDR of HepG2 cells was paralleled by increase in cell viability (Fig. 2A). The Rac inhibitor reduced the percentage of cells present in the subG1 fraction and mitigated the accumulation of doxorubicin-treated HepG2 cells in S-phase of the cell cycle (Supplementary Fig. S2). The cytoprotective effects of EHT184 also pertained to the epipodophyllotoxin etoposide (Fig. 2A), which is another type of topoisomerase II poison. In EHT1864 treated cells, the level of γH2AX was reduced up to 24 h after doxorubicin or etoposide treatment (Fig. 2B). EHT1864 did not block the increase in γH2AX following exposure to ionizing radiation (Fig. 2B), showing that the inhibitory effect of EHT1864 on the DDR is agent specific. Furthermore, EHT1864 attenuated the doxorubicin-induced phosphorylation of p53 (pp53) as well as of the stress kinases p38 (pp38) and JNK (pJNK) but not Akt (pAkt) (Fig. 2C). It also caused an increase in the constitutive expression of the anti-apoptotic factor survivin and mitigated the proteolytic cleavage of caspase 7 as observed 24 h after doxorubicin treatment (Fig. 2C). In line with the viability and γH2AX data, EHT1864 also reduced the amount of DNA strand breaks following doxorubicin and etoposide treatment as analyzed by the alkaline comet assay (Fig. 2D).

p53 plays a pivotal role in the DDR by regulating checkpoint activation, DNA repair and apoptosis [23]. P53 becomes activated in response to DSBs or stalled replication forks [23] by phosphorylation, which is catalyzed by the PI3-like kinases AMT/ATR, the key regulators of the DDR. Bearing in mind the major function of p53 in the regulation of genotoxic stress responses, we wondered whether it interferes with the geno- and cytoprotective effects evoked by the Rac inhibitor EHT1864. For this reason, Hep3B cells, which lack p53 [49], were included in our study. In line with literature [49], p53 protein stabilization following doxorubicin exposure was not found in Hep3B cells (Fig. 1A). Similar to what we observed in HepG2 cells, EHT1864 also protected Hep3B cells from the cytotoxic effects of doxorubicin and etoposide (Fig. 3A), reduced the increase in γH2AX levels up to 24 h following treatment with topo II poisons (Fig. 3B) and diminished DNA strand break formation (Fig. 3C). Based on these data we hypothesized that EHT1864-mediated protection of hepatoma cells from the geno- and cytoprotective effects of topo II poisons is independent of p53. This hypothesis was supported by the finding that Rac inhibition in HepG2 cells, which harbor wildtype p53, leads to a reduction of doxorubicin-stimulated H2AX phosphorylation even in the presence of the p53 inhibitor pifithrin (Fig. 3D). Pifithrin pre-treatment did not affect H2AX phosphorylation on its own (data not shown). Noteworthy, while EHT1864 and pifithrin did not provoke appreciable cytotoxicity on their own, co-treatment with EHT and pifithrin resulted in a massive reduction of cell viability (Fig. 3E).

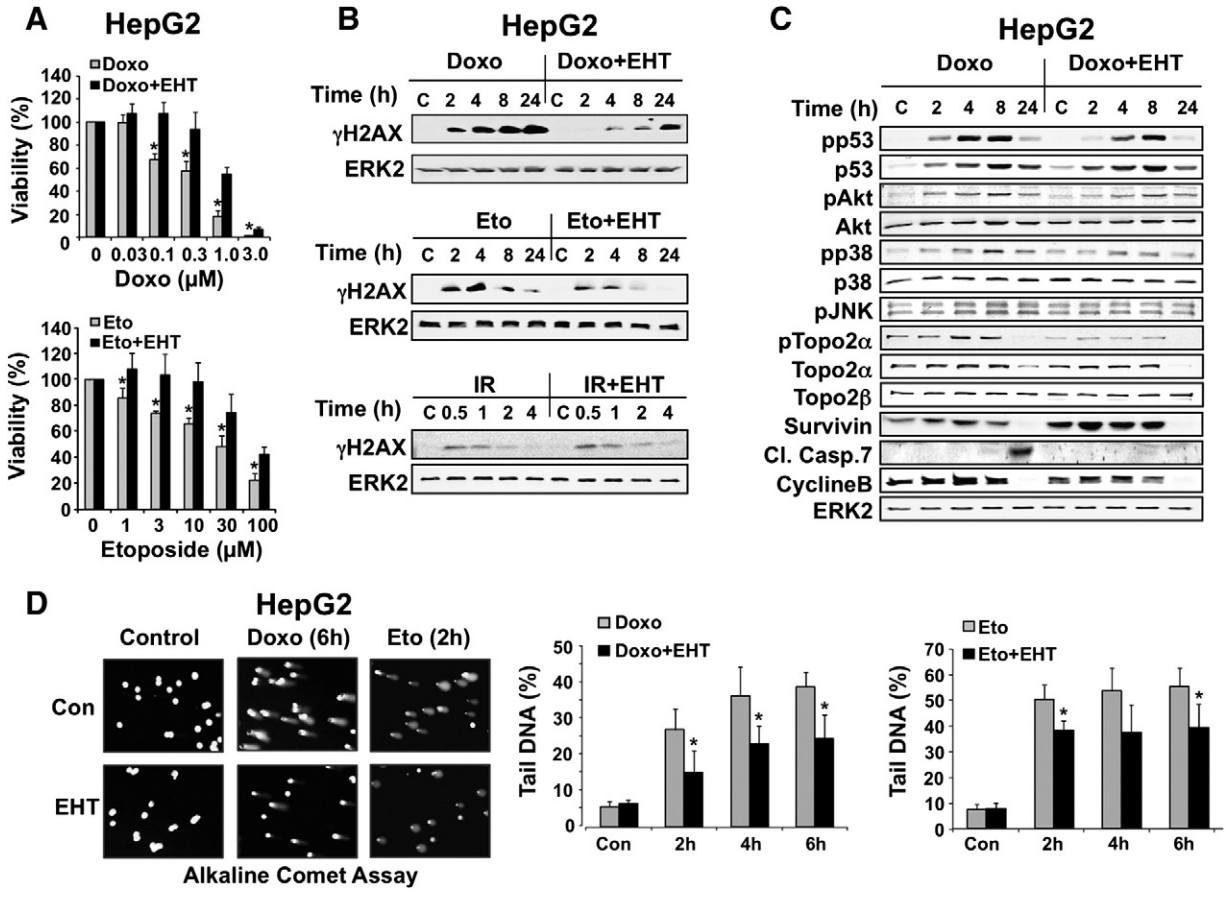

Fig. 2. EHT1864 protects human hepatoma cells (HepG2) from the geno- and cytotoxic effects of topoisomerase II inhibitors. A: Cells were pretreated or not with EHT1864 (10 μM) (EHT) for 1 h before doxorubicin (Doxo) or etoposide (Eto) was added at different concentrations as indicated. Cell viability was analyzed after incubation period of 72 h using the WST assay. Relative viability of untreated control was set to 100%. Data shown are the mean ± sd from 3 independent experiments each performed in triplicate. *$p \leq 0.05$. B: After pretreatment with EHT1864 (10 μM) for 1 h, cells were exposed to the topoisomerase II poisons doxorubicin (1 μM) (Doxo) or etoposide (10 μM) (Eto). For control, cells were treated with ionizing radiation (IR) (10 Gy). Different time points (2–24 h) after treatment, the expression of S139 phosphorylated histone H2AX (γH2AX) was analyzed by western blot. Expression of ERK2 was monitored as protein loading control. C, untreated control. C: Logarithmically growing HepG2 cells were pretreated with EHT1864 (EHT), followed by addition of doxorubicin (Doxo) as described under B. Up to 24 h after doxorubicin treatment, protein expression of a selected subset of factors was analyzed. C, untreated control. D: Cells that have been pretreated with EHT1864 (10 μM) for 1 h were exposed to doxorubicin (1 μM) (Doxo) or etoposide (10 μM) (Eto). After incubation periods of 6 h and 2 h for doxorubicin and etoposide, respectively, the level of DNA strand breaks was analyzed by the alkaline comet assay. Quantitative data shown in the histogram are the mean ± sd from 3 independent experiments. *$p \leq 0.05$.

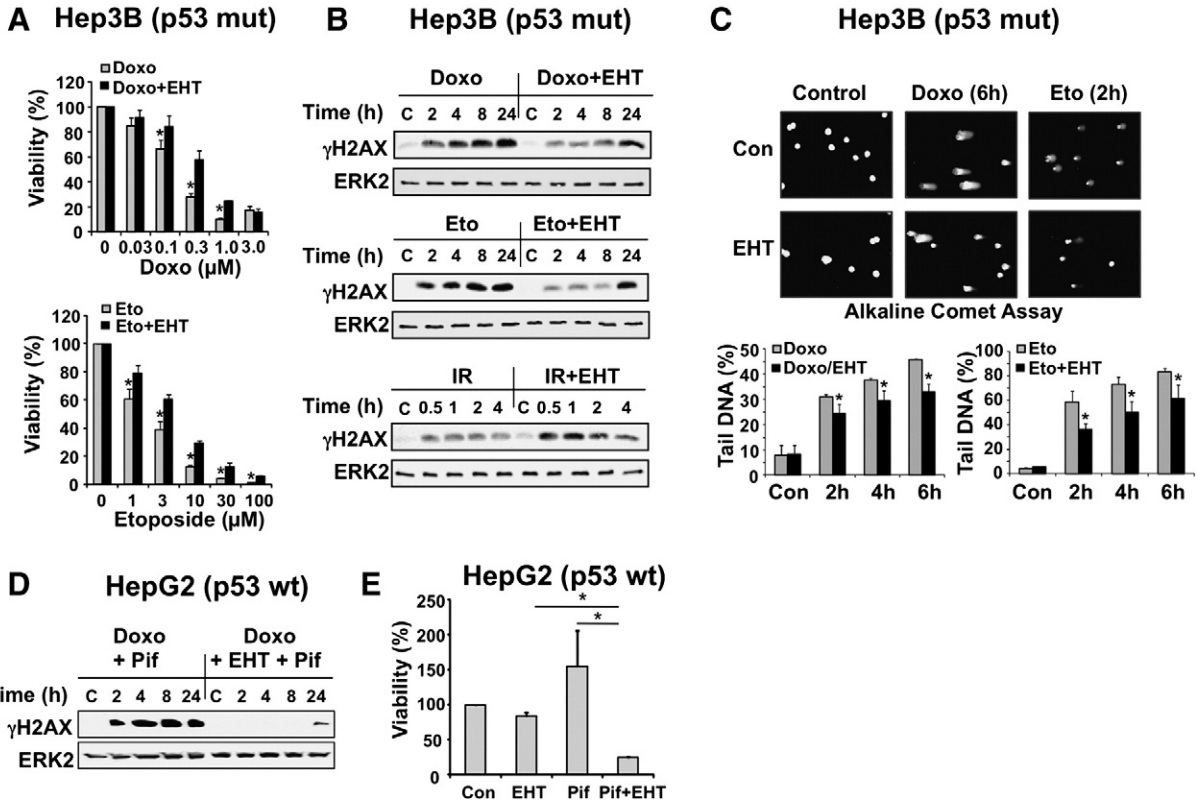

Fig. 3. EHT1864 protects p53 deficient human hepatoma cells (Hep3B) from the geno- and cytotoxic effects of topoisomerase II inhibitors. A: Logarithmically growing p53 deficient Hep3B cells were pretreated with EHT1864 (10 μM) for 1 h before doxorubicin (Doxo) or etoposide (Eto) was added at different concentrations as indicated. Cell viability was analyzed after incubation period of 72 h using the WST assay. Relative viability of untreated control was set to 100%. Data shown are the mean ± sd from 3 independent experiments each performed in triplicate. *$p \leq 0.05$. B: After pretreatment with EHT1864 (10 μM) for 1 h, Hep3B cells were exposed to the topoisomerase II poisons doxorubicin (1 μM) (Doxo) and etoposide (10 μM) (Eto). For control, cells were treated with ionizing radiation (IR) (10 Gy). Different time points (2–24 h) after treatment, the expression of S139 phosphorylated histone H2AX (γH2AX) was analyzed by western blot. Expression of ERK2 was monitored as protein loading control. C, untreated control. C: Hep3B cells that have been pretreated with EHT1864 (10 μM) for 1 h were exposed to doxorubicin (1 μM) (Doxo) or etoposide (10 μM) (Eto). After incubation periods of 6 h and 2 h for doxorubicin and etoposide, respectively, the level of DNA strand breaks was analyzed by the alkaline comet assay. Quantitative data shown in the histogram are the mean ± sd from 3 independent experiments. *$p \leq 0.05$. D, E: Following pretreatment of p53 wildtype HepG2 cells with the p53 inhibitory molecule pifithrin (Pif) (30 μM, 1 h) in the presence or absence of EHT1864 (10 μM), doxorubicin (1 μM) was added. After incubation period of 2–24 h, cells were harvested for the analysis of H2AX phosphorylation (γH2AX) (D). HepG2 cells were pretreated for 1 h with pifithrin (Pif) (30 μM) alone or in combination with EHT1864 (EHT) (10 μM). Cell viability was analyzed 72 h later. Data shown are the mean ± sd from three independent experiments each performed in triplicate (E). *$p \leq 0.05$.

Next we addressed the question whether the genoprotective effect of EHT1864 is restricted to a particular subpopulation of cells. To this end cells were scored with regard to the degree of doxorubicin-induced DNA strand breaks. As shown in Fig. 4A, EHT1864 predominantly reduced the frequency of cells that have been severely damaged by the anthracycline, i.e. cells with ≥40% of their DNA being in the tail (Fig. 4A). FACS-based cell cycle analysis revealed that cells present in S and G2 phase express the highest level of γH2AX, indicating that they contain the highest level of DNA damage (Fig. 4B). EHT1864 equally lowered doxorubicin-induced DNA damage in both S- and G2-phase cells (Fig. 4B). Taken together, the data show that inhibition of Rac signaling protects cells present in S- or G-phase from severe DNA damage following doxorubicin treatment.

3.3. Doxorubicin stimulated DDR can be suppressed by different types of protein kinase inhibitors and is independent of mechanisms of nuclear import

Next, we addressed the question as to the Rac downstream pathways that are required for activation of the DDR following doxorubicin exposure of HepG2 cells. To this end, a large variety of pharmacological inhibitors of protein kinases and other signaling molecules were investigated with respect to their potency to mitigate the doxorubicin-stimulated increase in γH2AX as EHT1864 does. Apart from EHT1864, which was included as internal control, a selected subset of 20 different inhibitors was used. These extensive analyses showed that a several pharmacological inhibitors were able to mimic the EHT1864 effect, i.e. to attenuate the doxorubicin-induced increase in γH2AX level by ≥50% (Fig. 5A, B and supplementary Table 2). Apart from NSC23766, which is another type of Rac inhibitor [45] blocking Rac1 activity in HepG2 cells as analyzed by GST-PAK pulldown assay (data not shown), inhibitors of stress-activated protein kinases/c-Jun-N-terminal kinases (SAPK/JNK), extracellular regulated kinases (ERK), p21-associated kinases (PAK), phosphoinositide-3-kinase (PI3K) as well as of EGFR receptor kinase, c-Abl and c-Src avoided the doxorubicin-stimulated increase in γH2AX (Fig. 5A and B). Moreover, the proteasome inhibitor MG132 and the nuclear export inhibitor ratjadone also showed inhibitory effects. By contrast, inhibitors of p38 kinase, Akt kinase, protein kinase CK2, cyclin dependent kinase 1 (CDK1), heat shock protein 90 kDa (Hsp90), NF-κB and inhibitor of nuclear import (ivermectin) were ineffective (Fig. 5A and B). The observation, that various kinase inhibitors had inhibitory potency indicates that multiple kinases are required for doxorubicin-stimulated DDR. Attenuation of doxorubicin-induced increase in γH2AX level by a given inhibitor was not always paralleled by corresponding reduction of pp53 levels. For instance, inconsistent effects were observed for inhibitors of JNK, CK2, CDK1, Hsp90 and nuclear import (Fig. 5A and B; Supplementary Table 2). Apparently, regulatory mechanisms of the DDR involved in the phosphorylation of H2AX and p53 are not identical.

Inhibition of upstream kinases of Rac, such as the tyrosine kinases EGFR, c-Abl or c-Src, was less effective in inhibiting doxorubicin-induced H2AX phosphorylation as compared to inhibition of downstream

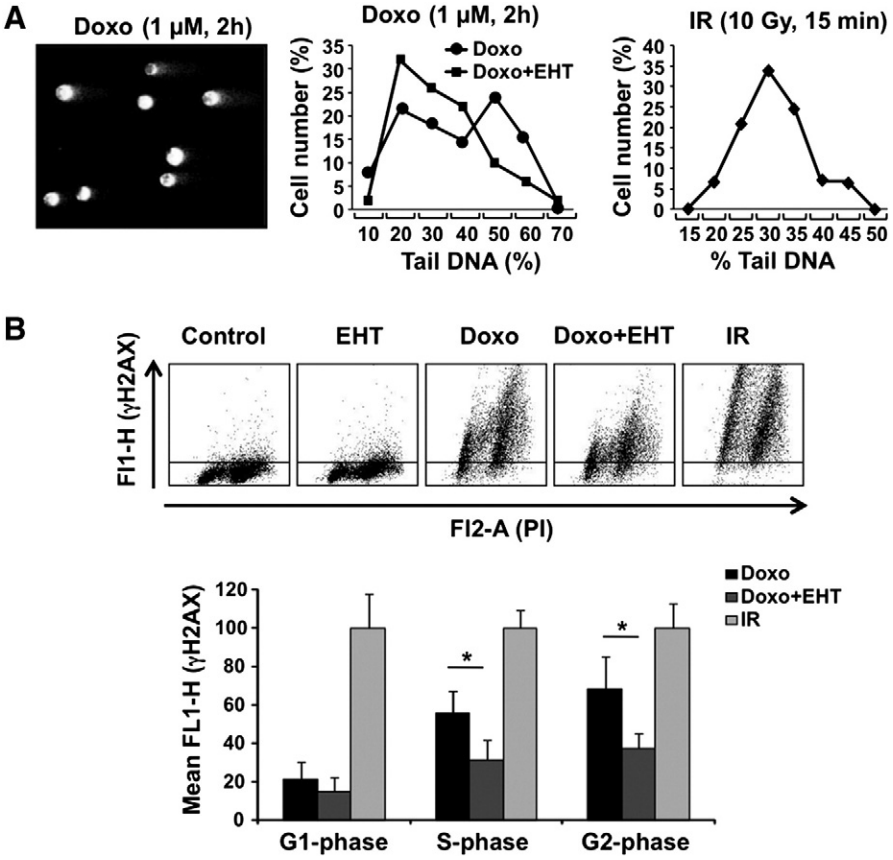

Fig. 4. EHT1864 protects HepG2 cells present in S- and G2-phase from severe DNA damage induced by doxorubicin. A: Logarithmically growing HepG2 cells were pretreated for 1 h with EHT1864 (10 μM), followed by exposure to doxorubicin (1 μM) for 2 h. For control cells were irradiated (10 Gy) and harvested 15 min after irradiation. DNA strand breaks were analyzed by the alkaline comet assay as described in the Materials and methods. The level of DNA damage was scored according to the % tail DNA (categories of 10–70%). Data shown are from three independent experiments with at least 50 nuclei having been analyzed per experiment. B: HepG2 cells were pretreated 10 μM EHT1864 for 1 h, followed by 2 h of doxorubicin (1 μM) treatment. For control cells were irradiated (10 Gy). After incubation period of 2 h (15 min in case of IR) γH2AX levels were determined by FACS analysis as described in the Materials and methods. To identify G1-, S- and G2-phase cells, nuclear DNA was co-stained by PI. Mean FL1-H values were determined for cells present in G1-, S- and G2-phase of the cell cycle and normalized to the IR control (IR value set to 100%). Quantitative data shown are the mean ± sd from four independent experiments performed in triplicate.

serine/threonine kinases such as JNK, ERK, PAK or PI3K. Presumably, inhibition of ERK is of particular relevance for the attenuation of doxorubicin-induced DDR by EHT1864, as ERK activates topoisomerase IIα [50,51] and affects doxorubicin resistance in breast cancer cells [52]. As topoisomerase II cleavable complexes are subject to proteasomal degradation [53], inhibition of the proteasome by MG132 is anticipated to prevent the degradation of topo II, eventually inhibiting the formation of DSBs. In line with this assumption, we found that MG132 attenuates the DDR, as reflected by a reduced phosphorylation of H2AX and p53 following doxorubicin treatment (Fig. 5A). Since MG132 stabilized the formation of the cleavable complex as analyzed by the TARDIS assay, whereas EHT1864 did not (Supplementary Fig. S3), we suggest that the inhibitory effect of EHT1864 on doxorubicin-induced DDR does not rest on an interference with the proteasomal degradation machinery.

Rac1 can translocate to the nucleus via importin α and binds to topoisomerase II [40]. Also topoisomerase II is imported into the nucleus by importin α and nuclear export of topoisomerase by CRM1 leads to doxorubicin resistance [54]. Bearing in mind these reports, we investigated whether the importin inhibitor ivermectin [55] attenuates doxorubicin-induced DDR to a similar extent as EHT1864 does. We found that ivermectin further promotes the doxorubicin-stimulated rise in γH2AX levels (Fig. 5B), rather than causing inhibition as speculated. This finding indicates that the mechanisms underlying the DDR inhibitory effect of EHT1864 are independent of alterations in the nuclear import of topoisomerases and Rac. The nuclear export inhibitor ratjadone partially blocked doxorubicin-stimulated DDR (Fig. 5B). Protein kinase CK2 phosphorylates topo IIα at several sites and was shown to affect drug resistance [56]. Based on this report we included the CK2 inhibitor resorufin [57] in our studies. Yet, resorufin did not mimic the inhibitory effect of EHT1864 on doxorubicin-induced DDR. Rather, it largely increased the level of γH2AX protein (Fig. 5A). Taken together, the results of the extensive inhibitor studies indicate that blockage of MAPK, rather than mechanisms of transport, contributes to inhibition of doxorubicin-stimulated DDR by EHT1864. It should be noted that inhibition of doxorubicin induced phosphorylation of H2AX and p53 by EHT1864, JNK inhibitor and MG132 was accompanied by attenuated autophosphorylation of ATM (Fig. 5C). The phosphorylation level of checkpoint kinase 2 (Chk2) was not affected by doxorubicin (Fig. 5C), showing the selectivity of doxorubicin-induced DNA damage responses.

3.4. Rac1 signaling modulates the phosphorylation status of topoisomerase IIα

Based on the aforementioned results, we hypothesized that topoisomerase II is phosphorylated by Rac-regulated protein kinases, resulting in conformational changes of topoisomerase II, which affects its interaction with topo II poisons. To substantiate this hypothesis, we investigated the effect of EHT1864 and the SAPK/JNK inhibitor SP60025, which showed a profound inhibition of doxorubicin-stimulated increase in γH2AX levels (see Fig. 5A), on the phosphorylation status of topoisomerase IIα using an antibody detecting S1106 phosphorylated protein. These analyses were performed in cytosolic and nuclear extracts of doxorubicin-treated cells and corresponding non-treated controls. EHT1864 caused a decrease in the level of S1106 phosphorylated nuclear topo IIα enzyme, which was accompanied by

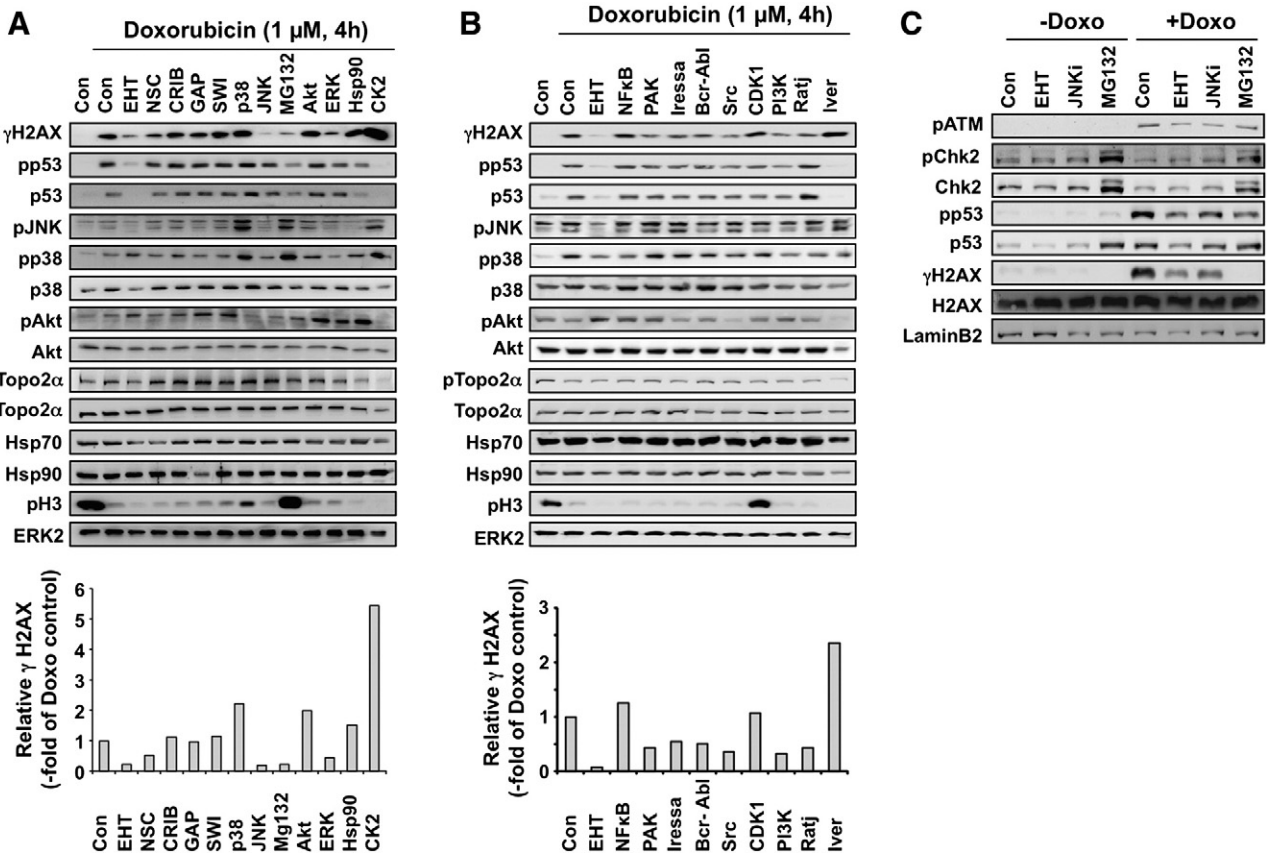

Fig. 5. A subset of protein kinase inhibitors mimic the inhibitory effect of EHT1864 on doxorubicin-induced DDR. A–B: Logarithmically growing HepG2 cells were pretreated for 1 h with different types of inhibitors, which are described in the Materials and methods. 4 h after addition of doxorubicin (1 μM), the expression level of the indicated proteins was analyzed by western blot. For densitometrical analysis, the relative amount of γH2AX in the doxorubicin treated cells was set to 1.0. Quantitative data are the mean from 2 independent experiments. C: Logarithmically growing HepG2 cells were pretreated for 1 h with EHT1864 (EHT), JNK inhibitor (JNKi) or proteasomal inhibitor (MG132) as described in the Materials and methods. 4 h after addition of doxorubicin (1 μM), phosphorylation status of key factors of the DDR, i.e. ATM, Chk2, p53 and H2AX was analyzed by western blot.

a decrease in the amount of nuclear Rac1 (Fig. 6A). Doxorubicin treatment did not affect the S1106 phosphorylation level of topo IIα. JNK inhibitor, which reduced the level of p-Jun as expected, had only marginal influence on the amount of p-Topo IIα protein (Fig. 6A). Following doxorubicin treatment, the level of nuclear Rac1 increased, which again was blocked by EHT1864 and the JNK inhibitor (Fig. 6A). Taken together, the data indicate that nuclear function of Rac1 is involved in regulating S1106 phosphorylation of topo IIα.

Phosphorylation of topo II at S1106 is reported to be catalyzed by different types of protein kinases, in particular protein kinase CK1 [44] and protein kinase CK2 [56]. As CK2 inhibition failed to block activation of the DDR following doxorubicin exposure (see Fig. 5A), we hypothesized

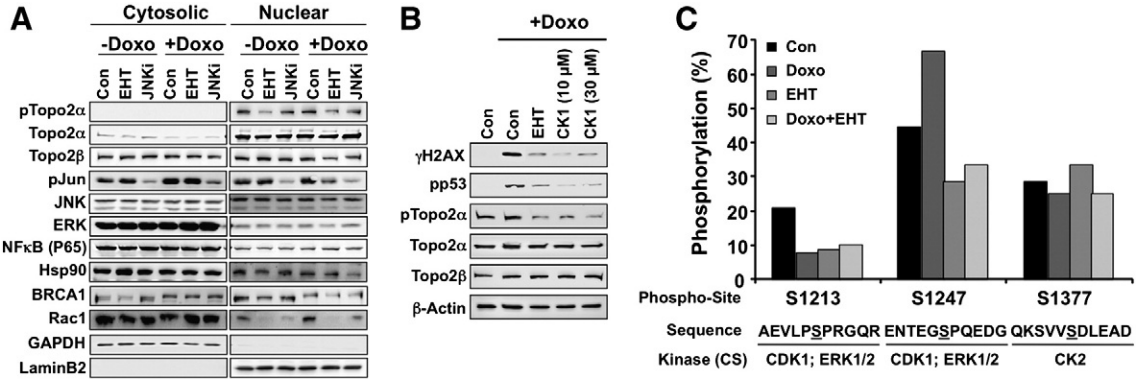

Fig. 6. EHT1864 treatment results in a reduced S1106 phosphorylation of topoisomerase IIα and a reduced nuclear import of Rac1. A: Logarithmically growing HepG2 cells were pretreated or not with EHT1864 (10 μM) (EHT) or the JNK inhibitor SP60025 (10 μM) (JNKi) for 1 h. Afterwards, doxorubicin (1 μM) was added for 4 h before cells were harvested for preparation of cytosolic and nuclear fractions as described in the Materials and methods. The expression of selected proteins present in either the cytosol or nucleus was analyzed by western blot. Purity of cytosolic and nuclear extracts was checked by analyzing the expression of GAPDH and lamin B2, respectively. B: Logarithmically growing HepG2 cells were left untreated or were pretreated with the CK1 inhibitor IC261 (10 μM or 30 μM) for 1 h. Afterwards, doxorubicin (1 μM) was added for 4 h before cells were harvested for western blot analysis using the indicated antibodies. C: Two hours after treatment of HepG2 cells with doxorubicin (1 μM) topoisomerase IIα protein was immunoprecipitated and phosphorylated amino acids were detected as described in the Materials and methods. The frequency of corresponding phospho-sites (S1213, S1247 and S1377) was calculated with respect to the corresponding unphosphorylated sites. The amino acid sequence containing the consensus sequence (CS) for particular protein kinases (CDK1, ERK1/2, CK2) is shown. EHT, pretreatment with EHT1864 (10 μM, 1 h).

that CK1 might be involved. Therefore, we additionally included the CK1 inhibitor IC261 in our study. The data obtained show that inhibition of CK1 blocked the doxorubicin-induced increase in γH2AX and pp53 levels and reduced the amount of S1106 phosphorylated topo IIα to a similar extent as EHT1864 (Fig. 6B). This finding indicates that CK1 is part of the Rac-regulated DDR stimulated by topo II poisons. In extension of the data obtained by use of S1106-phosphospecific topo IIα antibody, we immunoprecipitated topo IIα from untreated and treated cells and subjected the precipitate to mass spectrometrical analysis in order to identify additional phosphorylation sites affected by doxorubicin and/or EHT1864. A topo IIα peptide containing the S1106 phosphorylation was not detected. This might be due to the technical limitations of mass spectrometry. We also cannot rule out the possibility that the abundance of various phosphopeptides in the immunoprecipitate is influenced by selective depletion process resulting from covalent binding of topo IIα to DNA or enrichment mechanisms resulting from immunoprecipitation. Nevertheless, we found that treatment with both doxorubicin and EHT1864 reduced the basal phosphorylation of topo IIα at position S1213, whereas phosphorylation of S1377 remained unchanged (Fig. 6C). S1247 phosphorylation was slightly increased following doxorubicin exposure. Notably, EHT1864 mitigated both basal and doxorubicin-stimulated S1247 phosphorylation (Fig. 6C). Both S1213 and S1247 are present within a consensus sequence for MAPK kinases and CDK1, while S1377 is within a consensus sequence of CK2. Whereas S1106 phosphorylation of topo IIα has been related to a reduced cleavable complex formation [44], S1213 and S1247 phospho-sites have been associated with the regulation of G2/M-phase [58].

Deduced from the mass spectrometry-based data together with the western blot-based results obtained with the kinase inhibitors, we suggest that Rac-dependent MAPK-mediated phosphorylation of topo IIα at S1213 and S1247 as well as CK1-catalyzed phosphorylation of S1106 are of particular relevance for the interaction of topo II with its poisons. While the interplay of Rac with stress kinases is well known [4,5], it is not fully clear how Rac1 and CK1 can interact in the context of doxorubicin-induced DDR. Noteworthy, it has been shown that CK1 prevents activation of Rac, thereby facilitating non-canonical Wnt signaling pathways [59,60].

As the inhibitory effect of EHT1864 on doxorubicin-induced DDR likely rests on a reduced formation of the topo II-DNA cleavable complex [37], we investigated the influence of the various protein kinase inhibitors on the cleavable complex formation using the band-depletion assay [44]. Etoposide largely stimulates the covalent binding of topo II to the DNA, which is reflected by a large decrease in topo II protein level detectable by western blot analysis (Fig. 7). EHT1864 partially prevented the decrease in topo IIα levels (Fig. 7), indicating that Rac inhibition disables the formation of the cleavable complex. Bearing in mind that a very high dose of etoposide is used in the band-depletion assay, complete protection by EHT1864 cannot be expected. Apart from EHT1864, inhibition of a subset of protein kinases such JNK, ERK PI3K as well as CK1 also partially reduced the formation of the cleavable complex following etoposide treatment (Fig. 7). These results are in line with the hypothesis that Rac-regulated protein kinases, in particular MAPK and CK1, have a permissive role in the DDR evoked by topo II poisons.

3.5. EHT1864 rescues doxorubicin-induced inhibition of Hsp90–topo IIα interaction

Apart from Rac-regulated phosphorylation of topoisomerase II additional mechanisms might contribute to the observed genoprotective effect of EHT1864. Interaction of Hsp90 and topo IIα was reported to affect doxorubicin resistance [47,61]. As we did not find alterations in the expression level of topo IIα and Hsp90 protein in response to doxorubicin or EHT1864 treatment (see Fig. 1), we hypothesized that posttranslational mechanisms may regulate interactions between these proteins.

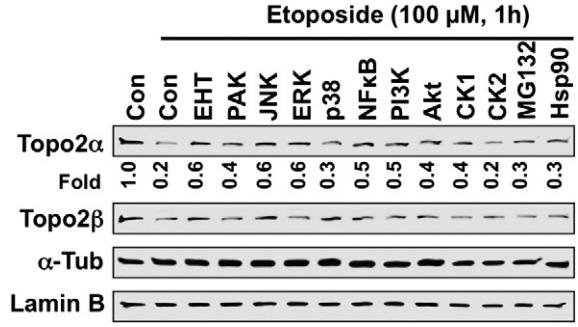

Fig. 7. Effect of EHT1864 and various protein kinase inhibitors on topo II-DNA complex formation as analyzed by the band-depletion assay. Logarithmically growing HepG2 cells were preincubated for 1 h with different types of inhibitors, which are described in the Materials and methods. Afterwards, cells were treated with a high dose of the topo II poison etoposide (100 µM). After incubation period of 1 h the protein level of the indicated proteins was investigated by western blot analysis. Decrease in the level of topo II proteins (=band-depletion) is indicative of the formation of the topo II-DNA cleavable complex. For quantitative densitometrical analysis, the relative topo IIα protein level in untreated control cells was set to 1.0. The filter was reprobed with α-tubulin and lamin B specific antibodies for protein loading control.

In order to address this question, co-immunoprecipitation experiments were performed. We observed that Hsp90 co-precipitates with Rac1 and that Rac1–Hsp90 interaction is reduced by both EHT1864 and doxorubicin treatment (Fig. 8A). Hsp90 also co-precipitates with topo IIα and Hsp90–topo IIα interaction is blocked by doxorubicin (Fig. 8B). This effect of doxorubicin is rescued by ETH1864 (Fig. 8B), indicating that Rac inhibition stabilizes the interaction between topo IIα and Hsp90. Hsp90 is also known to inhibit the SAPK/JNK regulatory kinase

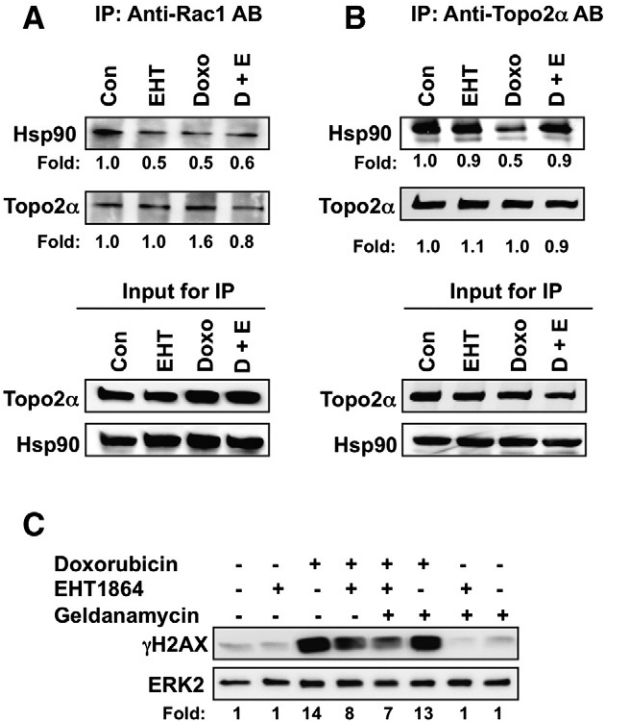

Fig. 8. Effect of EHT on Hsp90–topo IIα interaction. A: HepG2 cells pretreated or not wit EHT1864 (10 µM) (EHT) for 1 h were exposed to 1 µM doxorubicin (Doxo) for 2 h. Afterwards, immunoprecipitation (IP) was performed with anti-Rac1 and anti-topo IIα antibody. Co-immunoprecipitated proteins (Hsp90 and topo IIα) were detected by western blot analysis as described in the Materials and methods. The lower part of the figure shows that identical amount of protein was used for IP experiments (input for IP). B: HepG2 cells were treated with EHT1864 and doxorubicin as described under A. In addition, cells were co-treated or not with the Hsp90 inhibitor geldanamycin before doxorubicin exposure. S139 phosphorylation of H2AX was analyzed by western blot. Relative amount of γH2AX was set to 1.0 in untreated control (Con).

ASK1 [62]. As SAPK/JNK inhibition blocked the doxorubicin-induced phosphorylation of H2AX (see Fig. 5A), we explored the influence of the Hsp90 inhibitor geldanamycin on S139 phosphorylation of H2AX following doxorubicin treatment. Inhibition of Hsp90 by geldanamycin did not have major effects on the level of γH2AX following treatment with doxorubicin with or without EHT1864 co-treatment (Fig. 8C). Taken together, the findings indicate that binding of Hsp90 to topo IIα, which causes sequestration of topo IIα, is not of major relevance for the inhibitory effect of EHT1864 on doxorubicin-stimulated DDR.

Taken together, the data show that pharmacological inhibition of Rac signaling blocks the DDR induced by topo II poisons in a cell type-specific manner. Attenuation of the topo II poison-stimulated DDR under situation of Rac inhibition is independent of p53, Hsp90–topo II interaction and the nuclear export/import of topo II. Modification of topo IIα by phosphorylation is known to be important for its stability, nuclear localization, decatenation activity and DNA binding activity. Based on our data we suggest that a nuclear function of Rac is required for phosphorylation of topo II, which in turn is a prerequisite for the activation of the DDR by topo II poisons. We hypothesize that inhibition of Rac signaling by EHT1864 disables the phosphorylation of topo IIα at S1106, S1213 and S1247, which are catalyzed by multiple protein kinases, including MAPK and CK1. Lack of these phospho-sites results in structural alterations of the topo II protein that hampers the interaction with its poisons. In consequence, the formation of the topo II-DNA complex is impeded, which in turn attenuates the formation of DSBs and, ultimately, the activation of the DDR.

Disclosure of potential conflicts of interest

The authors declare that there are no potential conflicts of interest.

Grant support

This work was supported by the Deutsche Forschungsgemeinschaft (DFG Fr 1241/8-1).

Acknowledgement

We would like to thank Lena Schumacher for excellent technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.08.016.

References

[1] S. Aznar, J.C. Lacal, Rho signals to cell growth and apoptosis, Cancer Lett. 165 (2001) 1–10.
[2] G.M. Bokoch, Regulation of cell function by Rho family GTPases, Immunol. Res. 21 (2000) 139–148.
[3] A. Hall, Rho GTPases and the actin cytoskeleton, Science 279 (1998) 509–514.
[4] A. Minden, A. Lin, F.X. Claret, A. Abo, M. Karin, Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs, Cell 81 (1995) 1147–1157.
[5] O.A. Coso, M. Chiariello, J.C. Yu, H. Teramoto, P. Crespo, N. Xu, T. Miki, J.S. Gutkind, The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway, Cell 81 (1995) 1137–1146.
[6] C.A. Jefferies, L.A. O'Neill, Rac1 regulates interleukin 1-induced nuclear factor kappaB activation in an inhibitory protein kappaBalpha-independent manner by enhancing the ability of the p65 subunit to transactivate gene expression, J. Biol. Chem. 275 (2000) 3114–3120.
[7] C.E. Canman, M.B. Kastan, Three paths to stress relief, Nature 384 (1996) 213–214.
[8] S. Aznar, P. Fernandez-Valeron, C. Espina, J.C. Lacal, Rho GTPases: potential candidates for anticancer therapy, Cancer Lett. 206 (2004) 181–191.
[9] G. Fritz, B. Kaina, Rho GTPases: promising cellular targets for novel anticancer drugs, Curr. Cancer Drug Targets 6 (2006) 1–14.
[10] G. Fritz, Targeting the mevalonate pathway for improved anticancer therapy, Curr. Cancer Drug Targets 9 (2009) 626–638.
[11] V. Haydont, C. Bourgier, M. Pocard, A. Lusinchi, J. Aigueperse, D. Mathe, J. Bourhis, M.C. Vozenin-Brotons, Pravastatin Inhibits the Rho/CCN2/extracellular matrix cascade in human fibrosis explants and improves radiation-induced intestinal fibrosis in rats, Clin. Cancer Res. 13 (2007) 5331–5340.
[12] J. Huelsenbeck, C. Henninger, A. Schad, K.J. Lackner, B. Kaina, G. Fritz, Inhibition of Rac1 signaling by lovastatin protects against anthracycline-induced cardiac toxicity, Cell Death Dis 2 (2011) e190.
[13] J.K. Liao, U. Laufs, Pleiotropic effects of statins, Annu. Rev. Pharmacol. Toxicol. 45 (2005) 89–118.
[14] S. Rao, M. Lowe, T.W. Herliczek, K. Keyomarsi, Lovastatin mediated G1 arrest in normal and tumor breast cells is through inhibition of CDK2 activity and redistribution of p21 and p27, independent of p53, Oncogene 17 (1998) 2393–2402.
[15] P. Caffario, F. Dammacco, A. Gernone, F. Silvestris, Statins activate the mitochondrial pathway of apoptosis in human lymphoblasts and myeloma cells, Carcinogenesis 26 (2005) 883–891.
[16] K. Itoh, K. Yoshioka, H. Akedo, M. Uehata, T. Ishizaki, S. Narumiya, An essential part for Rho-associated kinase in the transcellular invasion of tumor cells, Nat. Med. 5 (1999) 221–225.
[17] M.R. Graaf, D.J. Richel, C.J. van Noorden, H.J. Guchelaar, Effects of statins and farnesyltransferase inhibitors on the development and progression of cancer, Cancer Treat. Rev. 30 (2004) 609–641.
[18] G. Fritz, HMG-CoA reductase inhibitors (statins) as anticancer drugs (review), Int. J. Oncol. 27 (2005) 1401–1409.
[19] O. Fromigue, Z. Hamidouche, P.J. Marie, Blockade of the RhoA-JNK-c-Jun-MMP2 cascade by atorvastatin reduces osteosarcoma cell invasion, J. Biol. Chem. 283 (2008) 30549–30556.
[20] Q. Zhou, J.K. Liao, Pleiotropic effects of statins — basic research and clinical perspectives, Circ. J. 74 (2010) 818–826.
[21] J.L. Nitiss, DNA topoisomerases in cancer chemotherapy: using enzymes to generate selective DNA damage, Curr. Opin. Investig. Drugs 3 (2002) 1512–1516.
[22] D.J. Smart, H.D. Halicka, G. Schmuck, F. Traganos, Z. Darzynkiewicz, G.M. Williams, Assessment of DNA double-strand breaks and gammaH2AX induced by the topoisomerase II poisons etoposide and mitoxantrone, Mutat. Res. 641 (2008) 43–47.
[23] J.W. Harper, S.J. Elledge, The DNA damage response: ten years after, Mol. Cell 28 (2007) 739–745.
[24] P.L. Olive, Detection of DNA damage in individual cells by analysis of histone H2AX phosphorylation, Methods Cell Biol. 75 (2004) 355–373.
[25] W.P. Roos, B. Kaina, DNA damage-induced apoptosis: from specific DNA lesions to the DNA damage response and apoptosis, Cancer Lett. 332 (2012) 237–248.
[26] E.P. Rogakou, D.R. Pilch, A.H. Orr, V.S. Ivanova, W.M. Bonner, DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139, J. Biol. Chem. 273 (1998) 5858–5868.
[27] L.C. Kremer, E.C. van Dalen, M. Offringa, J. Ottenkamp, P.A. Voute, Anthracycline-induced clinical heart failure in a cohort of 607 children: long-term follow-up study, J. Clin. Oncol. 19 (2001) 191–196.
[28] A.L. Ferreira, L.S. Matsubara, B.B. Matsubara, Anthracycline-induced cardiotoxicity, Cardiovasc. Hematol. Agents Med. Chem. 6 (2008) 278–281.
[29] K. Seiter, Toxicity of the topoisomerase II inhibitors, Expert Opin. Drug Saf. 4 (2005) 219–234.
[30] A.M. Azarova, Y.L. Lyu, C.P. Lin, Y.C. Tsai, J.Y. Lau, J.C. Wang, L.F. Liu, Roles of DNA topoisomerase II isozymes in chemotherapy and secondary malignancies, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 11014–11019.
[31] Y.L. Lyu, J.E. Kerrigan, C.P. Lin, A.M. Azarova, Y.C. Tsai, Y. Ban, L.F. Liu, Topoisomerase IIbeta mediated DNA double-strand breaks: implications in doxorubicin cardiotoxicity and prevention by dexrazoxane, Cancer Res. 67 (2007) 8839–8846.
[32] S. Zhang, X. Liu, T. Bawa-Khalfe, L.S. Lu, Y.L. Lyu, L.F. Liu, E.T. Yeh, Identification of the molecular basis of doxorubicin-induced cardiotoxicity, Nat. Med. 18 (2012) 1639–1642.
[33] J. Ma, Y. Wang, D. Zheng, M. Wei, H. Xu, T. Peng, Rac1 signalling mediates doxorubicin-induced cardiotoxicity through both reactive oxygen species-dependent and -independent pathways, Cardiovasc. Res. 97 (2013) 77–87.
[34] J. Damrot, T. Nuebel, B. Epe, W.P. Roos, B. Kaina, G. Fritz, Lovastatin protects human endothelial cells from the genotoxic and cytotoxic effects of the anticancer drugs doxorubicin and etoposide, Br. J. Pharmacol. 149 (2006) 988–997.
[35] M. Mahmoudi, I. Gorenne, J. Mercer, N. Figg, T. Littlewood, M. Bennett, Statins use a novel Nijmegen breakage syndrome-1-dependent pathway to accelerate DNA repair in vascular smooth muscle cells, Circ. Res. 103 (2008) 717–725.
[36] G. Fritz, C. Henninger, J. Huelsenbeck, Potential use of HMG-CoA reductase inhibitors (statins) as radioprotective agents, Br. Med. Bull. 97 (2011) 17–26.
[37] S.C. Huelsenbeck, A. Schorr, W.P. Roos, J. Huelsenbeck, C. Henninger, B. Kaina, G. Fritz, Rac1 protein signaling is required for DNA damage response stimulated by topoisomerase II poisons, J. Biol. Chem. 287 (2012) 38590–38599.
[38] V. Haydont, O. Gilliot, S. Rivera, C. Bourgier, A. Francois, J. Aigueperse, J. Bourhis, M.C. Vozenin-Brotons, Successful mitigation of delayed intestinal radiation injury using pravastatin is not associated with acute injury improvement or tumor protection, Int. J. Radiat. Oncol. Biol. Phys. 68 (2007) 1471–1482.
[39] M. Yoshida, I. Shiojima, H. Ikeda, I. Komuro, Chronic doxorubicin cardiotoxicity is mediated by oxidative DNA damage-ATM-p53-apoptosis pathway and attenuated by pitavastatin through the inhibition of Rac1 activity, J. Mol. Cell. Cardiol. 47 (2009) 698–705.
[40] K. Sandrock, H. Bielek, K. Schradi, G. Schmidt, N. Klugbauer, The nuclear import of the small GTPase Rac1 is mediated by the direct interaction with karyopherin alpha2, Traffic 11 (2010) 198–209.
[41] C. Onesto, A. Shutes, V. Picard, F. Schweighoffer, C.J. Der, Characterization of EHT 1864, a novel small molecule inhibitor of Rac family small GTPases, Methods Enzymol. 439 (2008) 111–129.

[42] A. Shutes, C. Onesto, V. Picard, B. Leblond, F. Schweighoffer, C.J. Der, Specificity and mechanism of action of EHT 1864, a novel small molecule inhibitor of Rac family small GTPases, J. Biol. Chem. 282 (2007) 35666–35678.
[43] P.L. Olive, J.P. Banath, The comet assay: a method to measure DNA damage in individual cells, Nat. Protoc. 1 (2006) 23–29.
[44] A.G. Grozav, K. Chikamori, T. Kozuki, D.R. Grabowski, R.M. Bukowski, B. Willard, M. Kinter, A.H. Andersen, R. Ganapathi, M.K. Ganapathi, Casein kinase I delta/epsilon phosphorylates topoisomerase IIalpha at serine-1106 and modulates DNA cleavage activity, Nucleic Acids Res. 37 (2009) 382–392.
[45] Y. Gao, J.B. Dickerson, F. Guo, J. Zheng, Y. Zheng, Rational design and characterization of a Rac GTPase-specific small molecule inhibitor, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 7618–7623.
[46] C.R. Barker, J. Hamlett, S.R. Pennington, F. Burrows, K. Lundgren, R. Lough, A.J. Watson, J.R. Jenkins, The topoisomerase II-Hsp90 complex: a new chemotherapeutic target? Int. J. Cancer 118 (2006) 2685–2693.
[47] U. Sarangi, K.R. Paithankar, J.U. Kumar, V. Subramaniam, A.S. Sreedhar, 17AAG treatment accelerates doxorubicin induced cellular senescence: Hsp90 interferes with enforced senescence of tumor cells, Drug Target Insights 6 (2012) 19–39.
[48] B. Cha, J.W. Lim, K.H. Kim, H. Kim, HSP90beta interacts with Rac1 to activate NADPH oxidase in *Helicobacter pylori*-infected gastric epithelial cells, Int. J. Biochem. Cell Biol. 42 (2010) 1455–1461.
[49] M. Muller, S. Wilder, D. Bannasch, D. Israeli, K. Lehlbach, M. Li-Weber, S.L. Friedman, P.R. Galle, W. Stremmel, M. Oren, P.H. Krammer, p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs, J. Exp. Med. 188 (1998) 2033–2045.
[50] N.J. Wells, I.D. Hickson, Human topoisomerase II alpha is phosphorylated in a cell-cycle phase-dependent manner by a proline-directed kinase, Eur. J. Biochem. 231 (1995) 491–497.
[51] P.S. Shapiro, A.M. Whalen, N.S. Tolwinski, J. Wilsbacher, S.J. Froelich-Ammon, M. Garcia, N. Osheroff, N.G. Ahn, Extracellular signal-regulated kinase activates topoisomerase IIalpha through a mechanism independent of phosphorylation, Mol. Cell. Biol. 19 (1999) 3551–3560.
[52] L. Smith, M.B. Watson, S.L. O'Kane, P.J. Drew, M.J. Lind, L. Cawkwell, The analysis of doxorubicin resistance in human breast cancer cells using antibody microarrays, Mol. Cancer Ther. 5 (2006) 2115–2120.
[53] Y. Mao, S.D. Desai, C.Y. Ting, J. Hwang, L.F. Liu, 26S proteasome-mediated degradation of topoisomerase II cleavable complexes, J. Biol. Chem. 276 (2001) 40652–40658.
[54] J.G. Turner, D.C. Marchion, J.L. Dawson, M.F. Emmons, L.A. Hazlehurst, P. Washausen, D.M. Sullivan, Human multiple myeloma cells are sensitized to topoisomerase II inhibitors by CRM1 inhibition, Cancer Res. 69 (2009) 6899–6905.
[55] K.M. Wagstaff, H. Sivakumaran, S.M. Heaton, D. Harrich, D.A. Jans, Ivermectin is a specific inhibitor of importin alpha/beta-mediated nuclear import able to inhibit replication of HIV-1 and dengue virus, Biochem. J. 443 (2012) 851–856.
[56] K. Chikamori, D.R. Grabowski, M. Kinter, B.B. Willard, S. Yadav, R.H. Aebersold, R.M. Bukowski, I.D. Hickson, A.H. Andersen, R. Ganapathi, M.K. Ganapathi, Phosphorylation of serine 1106 in the catalytic domain of topoisomerase II alpha regulates enzymatic activity and drug sensitivity, J. Biol. Chem. 278 (2003) 12696–12702.
[57] I.S. Sandholt, B.B. Olsen, B. Guerra, O.G. Issinger, Resorufin: a lead for a new protein kinase CK2 inhibitor, Anticancer Drugs 20 (2009) 238–248.
[58] M. Nousiainen, H.H. Sillje, G. Sauer, E.A. Nigg, R. Korner, Phosphoproteome analysis of the human mitotic spindle, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 5391–5396.
[59] V. Bryja, A. Schambony, L. Cajanek, I. Dominguez, E. Arenas, G. Schulte, Beta-arrestin and casein kinase 1/2 define distinct branches of non-canonical WNT signalling pathways, EMBO Rep. 9 (2008) 1244–1250.
[60] L. Cajanek, R.S. Ganji, C. Henriques-Oliveira, S. Theofilopoulos, P. Konik, V. Bryja, E. Arenas, Tiam1 regulates the Wnt/Dvl/Rac1 signaling pathway and the differentiation of midbrain dopaminergic neurons, Mol. Cell. Biol. 33 (2013) 59–70.
[61] C.R. Barker, A.V. McNamara, S.A. Rackstraw, D.E. Nelson, M.R. White, A.J. Watson, J.R. Jenkins, Inhibition of Hsp90 acts synergistically with topoisomerase II poisons to increase the apoptotic killing of cells due to an increase in topoisomerase II mediated DNA damage, Nucleic Acids Res. 34 (2006) 1148–1157.
[62] R. Zhang, D. Luo, R. Miao, L. Bai, Q. Ge, W.C. Sessa, W. Min, Hsp90-Akt phosphorylates ASK1 and inhibits ASK1-mediated apoptosis, Oncogene 24 (2005) 3954–3963.